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APPLICATION NUMBER: *60/543,880*
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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Active Heterogeneous siRNA Mixtures

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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[ ] Other (specify)
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METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant claims small entity status. See 37 CFR 1.27.
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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[ ] Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully submitted,

SIGNATURE Harriet Strimpel

Date 02/12/04

REGISTRATION NO. 37,008 *(If appropriate)*

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Docket Number: NEB-239

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PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number NEB-239

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Number 1 of 1

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Practitioner's Docket No. NEB-239 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Tzertzinis et al.

Application No.: not yet assigned
Filed: herewith
For: Active Heterogeneous siRNA Mixtures

Group No.: N/A
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR UNITED STATES LETTERS PATENT

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Title: ACTIVE HETEROGENEOUS siRNA MIXTURES

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ACTIVE HETEROGENEOUS siRNA MIXTURES

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THIS IS A PROVISIONAL APPLICATION

BACKGROUND OF THE INVENTION

There is an increased demand for the generation of gene-specific inactivation reagents for functional genomics, for genes that are potential drug targets, and biological pathway investigations. Gene silencing double-stranded RNA fragments (siRNAs) have been used in many cases to specifically inactivate selected targets in mammalian cells. Major obstacles in such endeavors are the costly design methodology and discovery of effective siRNAs since individual siRNAs vary greatly in their efficiency for gene silencing due to a phenomenon generally termed position dependence effect. This requires the synthesis and testing of multiple siRNAs to identify the most effective ones, a process known as siRNA validation. These stages in the production of effective siRNAs ready for use raises several fold the cost and time required for obtaining a specific reagent for each target. Additionally chemical synthesis of RNA is inherently expensive.

Standard methodologies require the use of 20-150 nM of siRNA in each transfection for appreciable effects. Recent reports have, however, demonstrated that concentrations higher than 25 nM transfected into mammalian cells lead to non-specific effects known as "off-target" effects (Semizarov et al.) which illustrates the necessity of validating each siRNA.

This application relates to the generation of gene-specific mixtures of siRNA that can be used as ready-for-use reagents for gene inactivation studies.

Description of Embodiments

1. Cloned DNA suitable for generating double-stranded RNA substrates for heterogeneous siRNA mixtures

Segments of mRNA sequence were selected for an identified target preferably after a sequence comparison within a gene database. An algorithm was developed to scan for candidate gene sequences using a window of 16-21 bp and providing matching sequences in the database. (for example, the UNIGENE database). Regions of the target sequence showing absence of hits to other targets were preferably selected.

Although there is no particular limitation on segment size, in the examples herein segments were selected in the range of about 150-1000 bp for example, 200-400 bp long.

The selected segment was amplified with PCR primers made according to standard protocols for PCR primer design. For example, primers may include a T7 promoter sequence at the 5' end. Other sequences can be used in place of the T7 promoter to facilitate cloning to one of the double T7 promoter vectors (Litmus 28i, Litmus 38i, Litmus-U from NEB).

For Litmus U, the following primer sequences were used: gggaaagu and ggagacau, where u stands for uracil. After the PCR reaction, the amplified DNA product was cloned directly in Litmus U using the USER protocol (NEB). The cloned fragments were used for the production of dsRNA using HiScribe (NEB). dsRNA was prepared from DNA (see above for example) using HiScribe (NEB). The reaction mixture was incubated at 42 ^{o}C for three hours and the dsRNA was phenol extracted, and dialyzed.

2. Generation and purification of hsiRNA

21-22 bp hsiRNA was typically produced from an RNAse III digestion in the presence of manganese ions (see US patent application 10/622,240 incorporated by reference) and (Shortcut RNA kit from NEB). ShortCut siRNA is a highly potent mixture of 21-22 bp dsRNA that is processed from a large gene-specific dsRNA (150-1500 bp) by RNase III in the presence of manganese buffer, the sequences of which collectively span the entire target RNA. The large dsRNA construct has been designed to be uniquely representative of the target gene with as little homology as possible to other areas of the genome.

The material obtained from the cleavage reaction was purified on a Pharmacia Source 15Q packed in an HR16/10 in a salt gradient using the following buffers:

Buffer A: 0 M NaCl, 20 mM Tris-Hcl pH 7.5 (25^{0} C), 0.5 mM EDTA.
Buffer B: 1 M NaCl, 20 mM Tris-Hcl pH 7.5 (25^{0} C), 0.5 mM EDTA.

The column was run on a Pharmacia AKTA FPLC system using the following program parameters:

Flow rate: 6 ml/min.
Start Concentration B: 0%.
Equilibration: 10 column volumes.
Load volume: ~100 ml.
Wash: 2 column volumes buffer A.
Fraction Size: 6 ml.
Target Concentration: 100%B.
Length of gradient: 20 column volumes.
Detection wavelength: 260nm

Pooling strategy/Source 15Q resolution

The Source Q resolves the major ds RNA digestion product (~21-22 base pair long) from smaller RNA fragments and RNAse III which elute from the column at lower salt prior to the collected peak, and from large dsRNA and substrate DNA which elute after the major peak at higher salt, observed by monitoring the flow conductivity. The siRNA containing fractions were pooled so as to avoid any larger size material, and any smaller RNA, while capturing the majority of the 21 bp siRNA. Fractions analyzed by non-denaturing PAGE confirmed this separation profile.

Examples of siRNA mixture preparations purified on the Source 15Q showed that the fragments of different hsiRNA were eluted at similar

salt concentrations making it possible to standardize the protocol for any desired fragment.

a. GFP siRNA fractions 30,31 eluted at 47.6% B and a conductivity of 43%.
b. Luciferase siRNA fractions 30-32 eluted at 48.3% B and a conductivity of 43.6%.
c. Litmus 28i polylinker siRNA fractions 29,30 eluted at 46%B and a conductivity of 41.4%.
d. Erk2 siRNA fractions 29-32 eluted at 47.6%B and a conductivity of 41.5%.

The Source 15Q pooled fractions of siRNA mixture from 3-4 fractions (usually 18-24 mls) were then dialyzed overnight against 2 liters of Storage Buffer (20 mM KCl, 10mM HEPES pH 7.0 (at 25^0 C), 0.5 mM EDTA, made with milli-Q or equivalent water). The dialyzed siRNA was frozen at –20 °C. Sterilize using passage through 0.2 µm filter and adjust the concentration to 150 ng/µl by dilution with sterile storage buffer before freezing for storage.

3. Endotoxin removal from heterogeneous siRNA mixture

For some applications it may be desirable to complete deplete the siRNA mixtures from any endotoxin (LPS) carried over from previous manipulations.

Endotoxin was measured using an LAL pyrochrome kit from Associates of Cape Cod Inc, Falmouth MA CAT# C0180. The endpoint method

listed in the product literature was used. The endotoxin was measured in Endotoxin Units (EU).

Examples of endotoxin levels measured in Source 15Q pools before purification are provided below:

a. AKT siRNA at approximately 0.5 mg/mL gave 16 EU/ml.
b. Luciferase siRNA gave 10.4 EU/ml.

Endotoxin levels can be significantly reduced by using a Pharmacia Source RPC directly after the Source 15Q protocol (3) to levels below 1EU.

The Source 15Q pool was loaded on a 3 ml Source RPC column.

Buffer A: 35 mM triethylamine (pH 7.0 with acetic acid at 25^0 C), 2% acetonitrile made with Milli-Q or equivalent water.
Buffer B: 100% acetonitrile.
The column was run on a Pharmacia AKTA FPLC system using the following program parameters:

Flow Rate: 2 ml/min.
Start Concentration B: 0%.
Equilibration: 5 column volumes A.
Load volume: ~50-100 ml.
Wash: 2 column volumes A.
Fraction Size: 1 ml.
Target Concentration: 20% B.
Length of gradient: 15 column volumes.

This protocol removes any contaminating endotoxin from the siRNA mixture which elutes at approximately 50% buffer B.
Examples of siRNA preparations purified on the Source RPC.

a. AKT siRNA fractions 27,28 eluted at 10.7% B.
b. Luciferase siRNA fractions 24-27 eluted at 10.6% B.

Fractions were transferred to 1.5 ml micro-centrifuge tubes and dried overnight in a spin vacuum without heat. Pellets were hydrated with storage buffer at room temperature.

Examples of endotoxin levels after the RPC purification:

a. AKT siRNA fraction 27 contained 0.18 EU/ml (800X reduction), fraction 28 contained 0.72 EU/ml (200X reduction).
b. Luciferase siRNA pool contained 0.062 EU/ml (168X reduction).

Both the Luciferase source Q- purified and RPC-purified siRNA mixtures are able to knock down by over 90% Luciferase expression transfected with siRNA mixtures at 1 nM in COS cells.

4. Preparation of a Kit
Applications of the kit include

- Gene silencing
- Target validation

The hsiRNA was formulated in: 20 mM KCl, 10 mM Na-HEPES (pH 7.0), 0.5 mM EDTA and was free of contaminating large molecular weight dsRNA, ssRNA, DNA, and protein.

The kit further optionally contains a transfection mixture (Transit-IT-TKO (Mirus Corp))

5. Transfection Protocol for hsiRNA into cells:

A very small amount of the hsiRNA is sufficient for effective silencing as compared to single sequence siRNA's. A starting concentration of 20 nM can be used which corresponds to 1 µl (10 pmol, 150 ng) of hsiRNA in 0.5 ml of transfection media.

a. Plate cells the day before so that the cell density is 40-60% confluent at the time of transfection.
b. Mix an appropriate amount of hsiRNA transfection reagent with serum free medium. Incubate at room temperature for 10-20 minutes.
c. Add an appropriate volume of hsiRNA mix (see table) to the diluted transfection reagent and incubate 10-20 minutes at room temperature to form the transfection complexes.
d. Dilute the complex with complete medium to the desired final culture volume for the plate size used (see table).
e. Aspirate the medium from the cell plate and replace with the diluted transfection complex.

Incubate cells 24-48 hours before analysis.

Examples for one transfection per well of the indicated size plates are shown in the table below. In the volumes shown the final siRNA mixture concentration is 20 nM.

Plate size	6	12	24	96
transfection reagent	6-10 µl	4-6 µl	2-4 µl	1-2 µl
Serum free medium	200 µl	100 µl	50 µl	25 µl
siRNA mix	4 µl	2 µl	1 µl	0.1 µl
Complete medium	800 µl	650 µl	450 µl	75 µl
Final volume	1000 µl	750 µl	500 µl	100 µl

6. Examples of Active Mixtures

Name:	CREB ShortCut siRNA mix
Concentration:	10 µM (150 ng/µl)
Size:	15 µg

Description: A heterogeneous mixture of 21-22 bp short interfering RNAs (siRNA) that induces effective silencing (RNAi) of the endogenous transcription factor CREB at concentrations of 20 nM and below in mammalian cell lines. CREB is a member of the leucine zipper

family of DNA binding proteins, binds as a homodimer to the cAMP-responsive element (CRE) and activates transcription in response to a variety of extracellular signals.

Western Blot:

AKT

CREB

- + ShortCut CREB siRNA (6.6 nM)

Western blot analysis of extracts from HeLa cells transfected with CREB ShortCut siRNA mix (+) or control siRNA (-). An antibody to the targeted protein CREB confirms silencing of protein expression, while a antibody against non-targeted AKT is used to control protein loading and to confirm siRNA specificity.

Source: A 360 bp DNA template derived from a CREB Mouse cDNA construct (coordinates 247-607, accession number m34356) was transcribed in vitro by T7 RNA polymerase using HiScribe to create double-stranded RNA (dsRNA). The dsRNA was processed by RNase III in the presence of manganese buffer (Shortcut) to produce a mixture of 21-22 bp siRNAs, and purified by column chromatography.

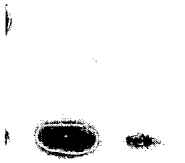
Name: p38 MAPK1 hsiRNA

Concentration: 10 µM (150 ng/µl)

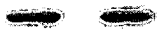
Size: 15 µg

Description: A heterogeneous mixture of 21-22 bp short interfering RNAs (siRNA) that induces effective silencing (RNAi) of the MAP kinase P38 at concentrations of 20 nM or less in mammalian cell lines. Supplied in: 20 mM KCl, 10 mM Na-HEPES (pH 7.0), 0.5 mM EDTA. Source: A 418 bp DNA template derived from a Human p38 cDNA construct (coordinates 10-419, accession number L35253) is transcribed by T7 RNA polymerase to create double-stranded RNA (dsRNA). RNase III cleaves the dsRNA in the presence of manganese buffer to 21-22 bp siRNA.

Western Blot:



p38 (target)



ATP citrate lyase (control)

\- + ShortCut p38 siRNA (6.6 nM)

Western blot analysis of extracts from HeLa cells transfected with p38 ShortCut siRNA mix (+) or non-targeted (-) control siRNA. An antibody to the targeted protein p38 confirms silencing of protein expression, while a non-targeted antibody against cs is used to control protein loading and to confirm siRNA specificity.

Name: p42/44 MAPK1 (ERK2) ShortCut siRNA mix

Concentration: 10 µM (150 ng/µl)
Size: 15 µg

Description: A heterogeneous mixture of 21-22 bp short interfering RNAs (siRNA) that induces effective silencing (RNAi) of the endogenous transcription factor ERK2 at concentrations of 20 nM and below in mammalian cell lines.

Source: A 283 bp DNA template derived from a Human Erk1 cDNA construct (coordinates 667-950, accession number NM_002745) is transcribed by T7 RNA polymerase to create double-stranded RNA (dsRNA). RNase III cleaves the dsRNA in the presence of manganese buffer to 21-22 bp siRNA.

Western Blot:

Western blot analysis of extracts from HeLa cells transfected with ERK2 ShortCut siRNA mix (+) or non-targeted (-) control siRNA. An antibody to the targeted protein ERK2 confirms silencing of protein expression, while a non-targeted antibody against Dynamin is used to control protein loading and to confirm siRNA specificity.

Table 1

CATEGORY	TARGET	*Acc. number*	Coordinates
kinases	Akt1	NM_005163	199-657
	Erk2	NM_002745	660-940
	MSK	AF074393	282-736
	p38	L35253	10-419
	IRS1	NM005544	1026-1713
	PKR	M35663	999-1499
	PTEN	NM_000314	1019-1445
transcription	CREB	M34356	247-601
Nuc. signaling	ERa	NM_000125	369-905
	ERb	NM_001437	587-1240
	DAX	NM_000475	1-249
	p53	NM_000546	717-915
	DNMT1	X69632-G-BPR2	2124-3235
	DnMT3B	AF331857	1150-1545
	DnMT3A	X63692.gb-pr2	1547-2388
	TRIP	L38810	1-445
	Rb	m15400.gb_pr1	2239-2755
	MeCP2	af030876.gb_pr	699-1011
Other	caspase3	p42574	1063-1496
	La	NM_003142	316-631
	FURIN	NM002569	1781-1990
Controls,	Lit28i polylinker	NEB#N3528S	2465-2600
gen. use	EGFP	U55763	596-1322
	RFP	AF272711	152-632
	FfLUC	U47295	747-1757
	Renilla	AF264722	3673-3951

Table 1 lists a series of target genes for which hsiRNA fragments have been or are being prepared from dsRNA having a sequence corresponding to the coordinates for the gene (cDNA) sequence contained in accession number of GenBank given above.